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Sympathetic Nerve Endings in the Pineal Gland Protect Against Acute Stress-Induced Increase in N-Acetyltransferase (EC 2.3.1.5.) Activity

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ABSTRACT. Injection of the antidepressant desmethylimipramine (DMI, 10 mg/kg) into intact rats or into rats in which the superior cervical ganglia had been decentralized caused a marked enhancement of the swimming stress-induced increase in pineal gland acetyl-CoA:serotonin N-acetyltransferase (N-acetyltransferase, EC 2.3.1.5.) activity. DMI is known to block uptake, the transport of catecholamines by nerve endings. It was found that DMI had no effect on enzyme activity in superior cervical ganglionectomized (SCGX) rats which were swimming-stressed. The pineal glands of these animals are devoid of nerve endings. In unstressed intact or unstressed surgically altered rats, injection of DMI caused only a minor increase in N-acetyltransferase activity, which was much smaller than that seen after stress.

After 5 h in organ culture sympathetic nerve endings within the pineal gland are still intact. At this time DMI treatment of pineal glands taken from intact rats shifted the dose-response curve for epinephrine (EPI) stimulation of N-acetyltransferase activity by two orders of magnitude, but caused only a slight change in the dose-response curve for isoproterenol, which is not taken up into nerve endings. In contrast, DMI treatment in organ culture had no effect on the dose-response curve for EPI in denervated pineal glands.

These results support the hypothesis that the response of pineal N-acetyltransferase activity to stimulation by stress is influenced by uptake$_1$. It would appear that in addition to terminating neuronal adrenergic transmission, this transport process physiologically protects the pineal gland against nontranssynaptic adrenergic stimulation. (*Endocrinology* 99: 840, 1976)

THE CIRCADIAN RHYTHM in the activity of pineal N-acetyltransferase, the enzyme which regulates large changes in the production of melatonin, is controlled by a transsynaptic adrenergic mechanism (1,2). At night, in response to central neural stimulation, norepinephrine is released from sympathetic nerve endings which have their cell bodies in the superior cervical ganglia (3). This sympathetic transmitter interacts with a beta$_1$ adrenergic receptor located on the pinealocyte membrane (1,2,4). Catecholamines released into the circulation in times of acute stress (2–2.5 h) can also increase the activity of pineal N-acetyltransferase (5,6). We find, however, that the N-acetyltransferase response to an acute stress in normal animals is remarkably small (6) compared with the 50- to 100-fold increase in activity seen either following injection of isoproterenol (8) or at night in the dark (9). It is striking that under similar stressful circumstances another physiological response regulated by circulating catecholamines, mobilization of glucose, is maximally stimulated. In addition an increase in the activity of several adrenal enzymes is observed (7).

A much larger N-acetyltransferase response to stress is seen in chemically sympathectomized animals (5) or animals in which the superior cervical ganglia have been removed (6). It has been suggested (5) that this increased response may be due to denervation supersensitivity (10). However, some of our earlier studies have indicated that normal animals which are stressed at times when their pineal gland should be supersensitive do not exhibit a substantially increased stress response (6). This has led to our speculation that pineal nerve endings play a protective role in acute stress (6), one

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Abbreviations used are as follows: N-acetyltransferase, acetyl-CoA: arylamine N-acetyltransferase (EC 2.3.1.5); DMI, desmethylimipramine; IMIP, imipramine; SCGX, superior cervical ganglionectomized; EPI, epinephrine.

in which they act as a sponge to remove catecholamines originating from the adrenal and other peripheral structures. In this manner the effective concentration of catecholamines in the perivascular space available to interact with the $beta_1$ adrenergic receptor is maintained at substimulatory concentrations. In the present report we have examined this proposal by treating stressed animals with the antidepressants desmethylimipramine (DMI) and imipramine (IMIP). These compounds are potent and specific inhibitors of "$uptake_1$," the transport mechanism involved in the concentration of catecholamines by adrenergic structures (11). Organ culture experiments with DMI were done to complement the *in vivo* studies.

Our results indicate that $uptake_1$, in addition to its well established role in terminating neuronal transmission (11), may also serve an equally important physiological role in some adrenergically innervated tissues of preventing stimulation by circulating catecholamines released during stress.

Materials and Methods

Male Sprague-Dawley rats (100–120 g, Zivic-Miller, Allison Park, Pa.) were used. Operative procedures were done by Zivic-Miller 21 days prior to experimental stress or to organ culture of pineal glands. Decentralization was accomplished by bilaterally excising a 2 mm section of the sympathetic chain caudal to each superior cervical ganglion. The ganglia were not manipulated directly. Unless otherwise stated, rats were housed for 5 days in light-tight chambers (light:dark 14:10) with the dark period starting at 1900 h. Food and water were provided *ad libitum* except during periods of stress, when only water was available.

Swimming stress experiments

All stress experiments were done in light between 1200–1600 h. Rats were placed in a container filled with water to a depth of approximately 35 cm. The animals were killed by decapitation and their pineal glands were removed immediately following the end of the stress period.

A preliminary experiment was performed to determine the water temperature range over which the response to swimming stress was consistent (Fig. 1). In subsequent swimming stress experiments rats were routinely placed in water adjusted initially to 31.3 C; water temperature dropped to 30.0 ± 0.2 C after a 2.5 h period.

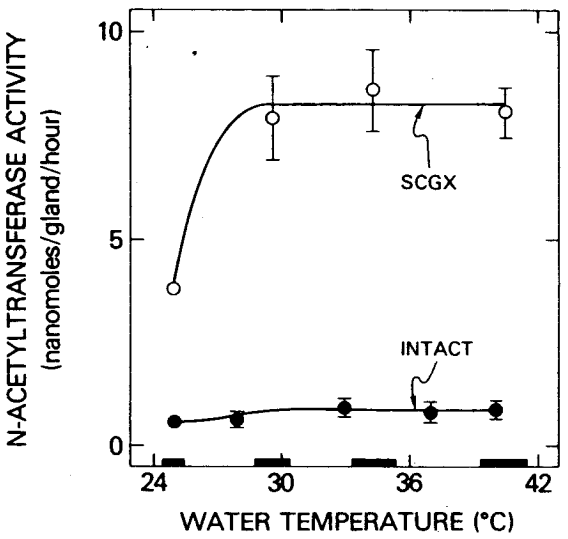

FIG. 1. Effect of water temperature on the magnitude of the swimming stress induced increase in pineal gland N-acetyltransferase activity in intact or superior cervical ganglionectomized (SCGX) rats. Groups of intact rats were swimming stressed for 2.5 h between 1300 and 1600 h. Water temperatures were maintained at 25 C, 28 C, 33 C, 37 C, or 39 C for the duration of the stress period by a circulating water heater (Ultra-Thermostat, Type 3, Lauda Instruments, Inc., Great Neck, N.Y.). SCGX rats were identically treated with the exception that water temperatures were not maintained constant by a circulating water bath during the stress period. The appropriate temperature was set at the start, then recorded at the end of the stress period. This latter procedure, unless otherwise stated, was used after we found that the water temperature in the soapstone sinks used for swimming stress dropped less than 2 C during the 2.5 h stress period. The black bars on the abscissa represent the temperature drop during the 2.5 h. Each point represents the mean (±SE) of the enzyme activity observed in 6 separate pineal glands.

A standard 2.5 hour stress period was chosen for several reasons. First, this time period was consistent with previous studies (5,6). Second, the peak response to acute stress, which occurred after 6 h in animals subject to superior cervical ganglionectomy (SGCX; Fig. 2), could be detected if it occurred earlier. Third, the earlier time point allowed for changes in the rate of

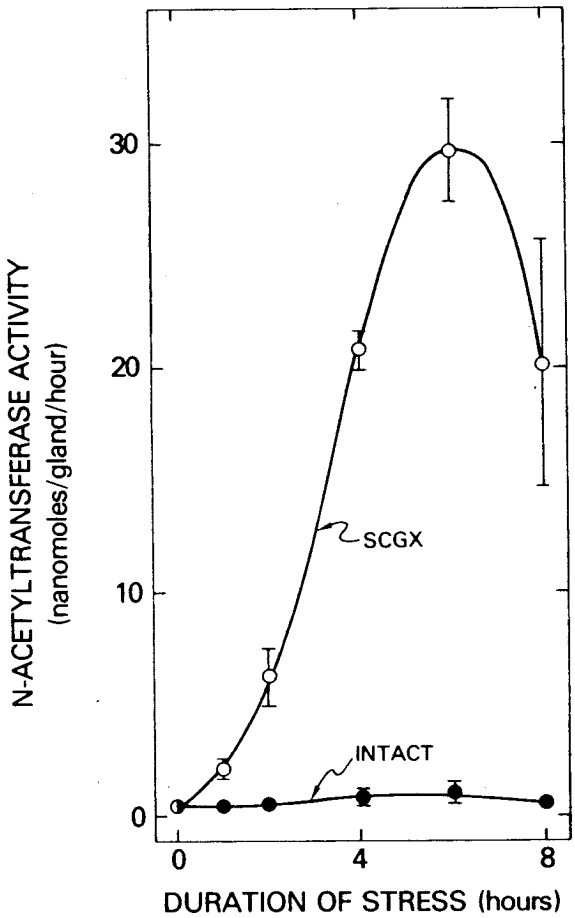

FIG. 2. Time course for increase in N-acetyltransferase activity in pineal glands of intact or SCGX rats subjected to swimming stress. Swimming stress was started at 1330 h at a water temperature of 31.3 C. Water temperature was readjusted in this experiment every 2 h to within 0.2 C of its initial value. Each point represents the mean (± SE) of the enzyme activity observed in 6 separate glands.

increase to be detected. Finally, the short stress period was necessary to concentrate on the acute aspects of this stress response.

In vivo *drug administration*

Drugs were dissolved in 0.85% NaCl to a final concentration of 100 mg/ml and the appropriate volume was injected subcutaneously.

Organ culture technique

Pineal glands were cultured as previously described (4). Pineal glands were immediately removed from culture at the end of drug treatment.

Enzyme assay

After pineal glands were removed from stressed animals or from organ culture they were immediately placed in 1.5 ml polyethylene assay tubes (Starstedt, Princeton, N.J.) cooled in solid CO_2. Glands were stored for 1–3 days at −75 C. N-Acetyltransferase activity was measured by a modification (12) of the method of Deguchi and Axelrod (13). Each pineal gland was sonicated in 100 μl of 0.5M sodium phosphate buffer, pH 6.8, containing 10 mM tryptamine HCl and 0.5 mM [1-^{14}C]acetyl-CoA (SA = 1 Ci/mol). After a 20 min incubation at 37 C, a 1 ml volume of chloroform was added to stop the reaction and to extract the product, N-[^{14}C]acetyltryptamine. The chloroform was taken to dryness; radioactivity was measured by conventional techniques.

Source of drugs

DMI was the generous gift of Geigy Pharmaceuticals (Ardsley, New York); IMIP (Eli Lilly, Indianapolis, Indiana) was the generous gift of Dr. P. Skolnick (NIAMDD). The sources of other compounds have been published (12).

Statistics

Data are presented as the mean ± SE. Statistical analysis was performed with Student's *t* test.

Results

1. Effect of DMI or IMIP on increase in N-acetyltransferase activity in intact rats subjected to swimming stress

Injection of DMI (10 mg/kg) into control intact rats caused an increased in enzyme activity from 0.2 ± 0.04 nmol/gland/h to 0.8 ± 0.17 nmol/gland/h (Fig. 3a). This may have been due to an accumulation of catecholamines in the region of the β-receptor of the pinealocyte. Such an accumulation could result from the combination of tonic discharge of afferent sympathetic neurons, as inferred from studies on the rate of turnover of [^{3}H]norepinephrine (14), and block-

ade of uptake$_1$. Another possibility is a large DMI-induced increase in circulating catecholamines. In addition, the brief stress of handling may have contributed to this effect. An increase in N-acetyltransferase activity in control intact rats treated with IMIP was not observed (Fig. 3b). This could represent an intrinsic difference between the potency or action of these drugs.

Enzyme activity in stressed intact rats was 0.6 ± 0.21 nmol/gland/h, a 3-fold increase over control intact rats. In the animals receiving DMI (10 mg/kg), enzyme activity after the 2.5 h stress period increased 10-fold over unstressed animals receiving DMI to 4.9 ± 0.58 nmol/gland/h.

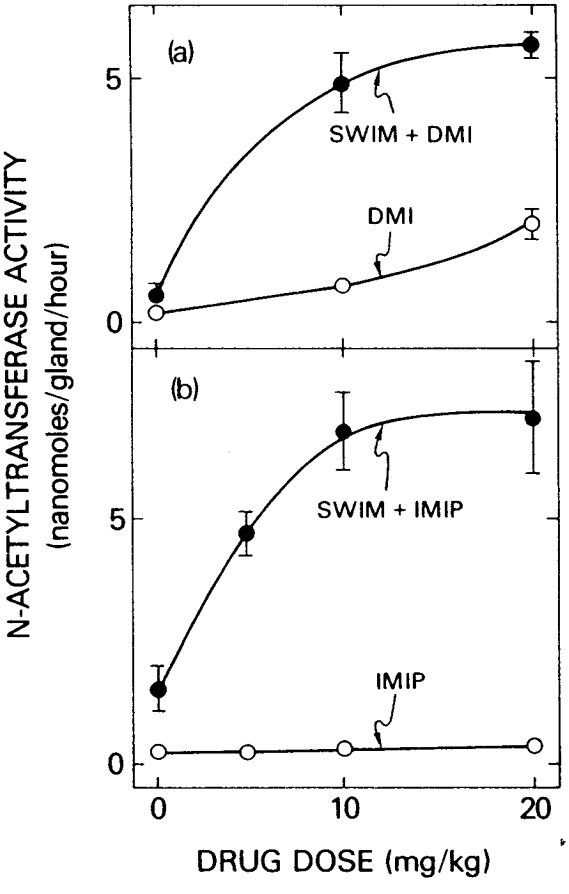

FIG. 3. Antidepressants and the stress-induced increase in pineal N-acetyltransferase activity in intact rats. a. Effect of DMI on the increase in pineal gland N-acetyltransferase activity in intact rats subjected to a 2.3 h swimming stress. b. Effect of IMIP on the increase in pineal gland N-acetyltransferase activity in intact rats subjected to a 2.5 h swimming stress. Each point represents the mean ± SE of the enzyme activity observed in 6 separate glands. The experiments presented in (a) and (b) were done on different days.

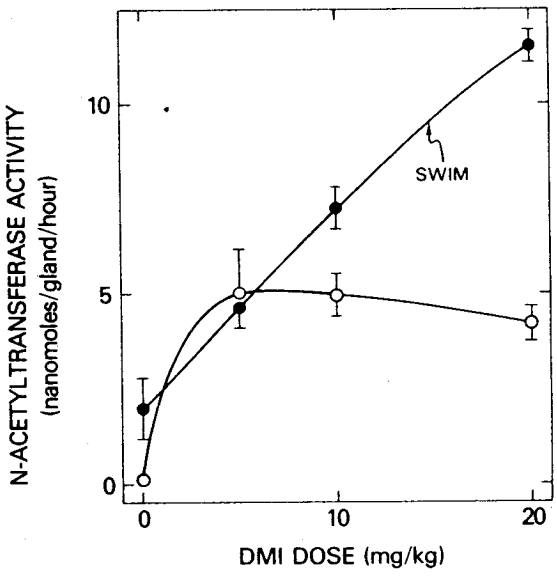

FIG. 4. Effect of DMI on the increase in pineal gland N-acetyltransferase activity in decentralized rats subjected to a 2.5 h swimming stress. Each point represents the mean ± SE of the enzyme activity observed in 6 separate glands.

N-Acetyltransferase activity in stressed animals treated with IMIP (10 mg/kg) increased to 7.1 ± 1.12 nmol/gland/h (Fig. 3b). In this experiment stressed animals not receiving IMIP had enzyme values of 1.5 ± 0.47 nmol/gland/h.

2. Effect of DMI on the increase in N-acetyltransferase activity in decentralized and SCGX rats subjected to swimming stress

We examined the effects of DMI on the stress response in animals chronically deprived of transsynaptic stimulation as a result of either decentralization or removal of the superior cervical ganglia (9). In both cases the pineal gland should be supersensitive (2). Only in the case of the decentralized animal, however, could the response to stress be influenced by nerve endings

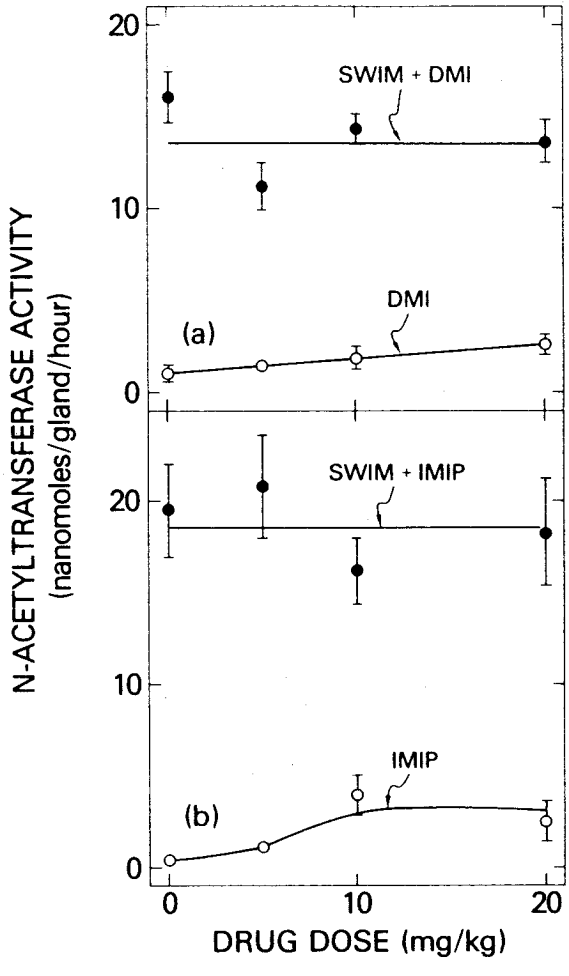

FIG. 5. Antidepressants and the stress-induced increase in pineal N-acetyltransferase in SCGX rats. a. Effect of DMI on the increase in pineal gland N-acetyltransferase activity in SCGX rats subjected to a 2.5 h swimming stress. b. Effect of IMIP on the increase in pineal gland N-acetyltransferase activity in SCGX rats subjected to a 2.5 h swimming stress. Each point represents the mean ± SE of the enzyme activity observed in 6 separate glands.

in the gland. If our proposal regarding the role of nerve endings is correct, the response of decentralized animals to stress should be enhanced by DMI; that of the ganglionectomized animals should not. This is essentially what was observed.

Swimming-stressed decentralized animals not receiving DMI had an enzyme activity of 2.0 ± 0.70 nmol/gland/h. After injection of DMI (20 mg/kg), the stress-induced increase in enzyme activity reached 11.5 ± 0.46 nmol/gland/h (Fig. 4). In the decentralized control animals, however, DMI also caused a marked increase in N-acetyltransferase activity. A value of 5.1 ± 1.12 nmol/gland/h was detected in animals receiving 5 mg/kg of DMI mg as compared with 0.1 ± 0.06 nmol/gland/h in the absence of DMI. This was much greater than the effect seen in intact rats, and it seems likely to have been a result of pineal supersensitivity in the decentralized animals. It may also reflect, however, an increase in the basal rate of turnover of norepinephrine in the nerve endings in these glands which might not receive a normal tonic inhibitory influence from central structures.

At all doses tested DMI or IMIP failed to enhance the stress-induced increase in pineal gland N-acetyltransferase activity in SCGX animals (Fig. 5a,b). Injection of DMI, however, did cause a significant increase in enzyme activity, from 1.0 ± 0.31 nmol/gland/h in the untreated SCGX animals to 2.6 ± 0.50 nmol/gland/h in animals treated with 20 mg/kg of DMI (Fig. 5a). In a separate experiment, IMIP (20 mg/kg) also caused an increase in unstressed animals from 0.4 ± 0.09 nmol/gland/h to 2.6 ± 1.11 nmol/gland/h (Fig. 5b). The greater effect of DMI and IMIP in unstressed decentralized rats as compared with unstressed SCGX rats may be a result of the local release of norepinephrine from nerve endings combined with the blocking effect of these compounds on neuronal uptake, a circumstance which would be absent in SCGX animals.

3. Effect of DMI treatment on the adrenergic stimulation of pineal N-acetyltransferase activity in organ culture

In the above *in vivo* experiments some of the observed effects of DMI could have been caused by the action of this drug on extra-pineal structures or by actions of DMI on pinealocytes. The following *in vitro* experiments were done to determine whether DMI could act directly on the pineal gland,

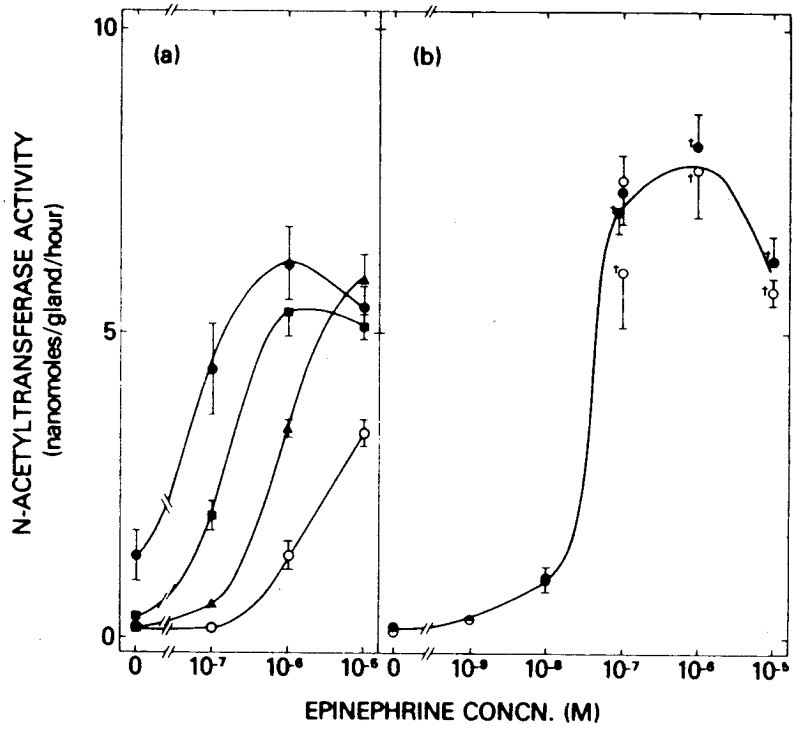

FIG. 6. Effect of DMI on N-acetyltransferase in pineal organ culture. a. Effect of DMI on the dose-response curve for EPI stimulation of N-acetyltransferase activity in pineal glands which had been cultured for 5 h following removal from intact rats. Pineal glands taken from intact rats were cultured for 5 h then treated for 2.5 h with EPI (10^{-5}M to 10^{-7}M) either alone (○) or in the presence of 10^{-5}M DMI (●), 10^{-6}M DMI (■), or 10^{-7}M DMI (▲). DMI treatment began 5 min before addition of EPI. b. Effect of DMI on the dose response curve for EPI stimulation of N-acetyltransferase activity in pineal glands which had been cultured for 48 h following removal from intact rats. Pineal glands taken from intact rats were cultured for 48 h then treated for 2.4 h with EPI (10^{-5}M to 10^{-9}M) either alone (○) or in the presence of 10^{-5}M DMI (●). This figure is a compilation of two separate experiments done on different days. †Signifies points determined in the first experiment. Each point represents the mean (±SE) of the enzyme activity determined in 4 separate glands.

and whether nerve endings were necessary for the potentiating action of this drug on the adrenergic stimulation of N-acetyltransferase activity (Fig. 6).

Pineal glands taken from intact rats were cultured for 5 h and then treated with DMI for 2.5 h. At the highest concentration used (10^{-5}M) DMI caused N-acetyltransferase activity to increase from 0.2 ± 0.04 nmol/gland/h to 1.3 ± 0.42 nmol/gland/h (Fig. 6a). This result is consistent with the explanation that the *in vivo* effect of DMI on N-acetyltransferase activity in unstressed intact or decentralized animals is due to local effects of the drug. DMI treatment also enhanced the increase in N-acetyltransferase activity in response to EPI stimulation, a result which is consistent with its effect in stressed intact rats. In the presence of 10^{-6}M DMI, the sensitivity to EPI was increased as indicated by a shift to the left of the dose-response curve by almost two orders of magnitude; a smaller shift was detected in the presence of 10^{-7}M DMI.

After 48 h in culture sympathetic nerve endings in the pineal gland have lost the majority of their dense-cored vesicles (15), and the uptake of [^{3}H]norepinephrine by the gland is reduced by 70% (Klein, D. C., and G. R. Berg, unpublished results), indicating that the nerve endings are no longer functional. At this time 10^{-5}M DMI alone did not cause an increase in enzyme activity and had no effect on the dose-response curve for EPI stimulation of N-acetyltransferase activity (Fig. 6b).

Pineal glands taken from SCGX rats are devoid of sympathetic nerve endings. If DMI were acting solely *via* nerve endings, then treatment with this drug would not be expected to cause any increase in sensitivity of the pineal gland to β-adrenergic stimulation. As shown in Fig. 7, treatment with 10^{-5}M DMI does not shift the dose-

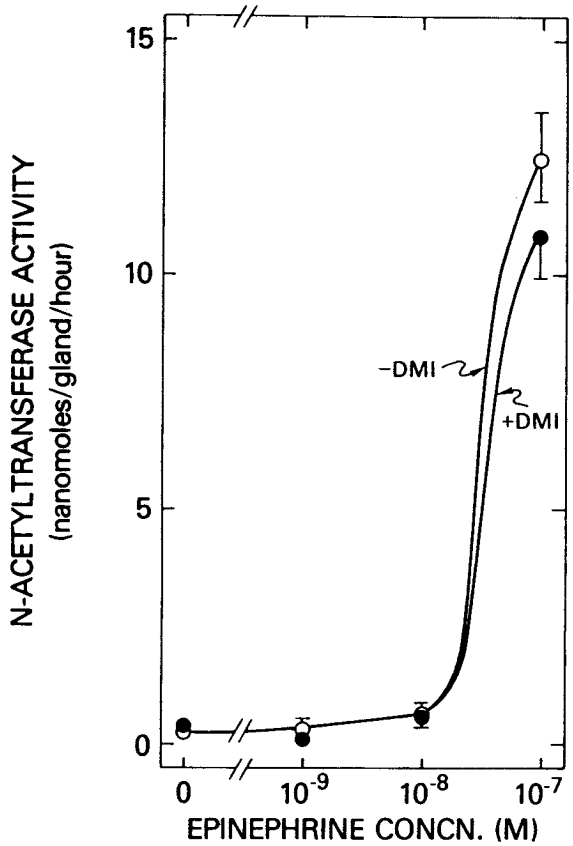

FIG. 7. Effect of DMI on the dose-response curve for EPI stimulation of N-acetyltransferase activity in pineal glands which had been cultured for 5 h following removal from SCGX rats. Pineal glands taken from SCGX rats were cultured for 5 h then treated for 2.5 h with EPI (10^{-7}M to 10^{-9}M) either alone (○) or in the presence of 10^{-5}M DMI (●). DMI treatment began 5 min before addition of EPI. Each point represents the mean (±SE) of the enzyme activity determined in 4 separate glands.

response curve for the EPI stimulation of N-acetyltransferase activity in denervated glands.

Another approach to the question of the mechanism of action of DMI was to examine its effect in the presence of the β-adrenergic agonist isoproterenol, which is a very poor substrate for uptake$_1$ (17). We found that treatment of pineal glands taken from intact rats (Fig. 8) with 10^{-6}M DMI caused only a very small shift in the dose-response curve for isoproterenol stimulation of N-acetyltransferase activity. A small increase in enzyme activity following treatment with DMI alone was again observed.

4. Effect of prolonged exposure to constant light on the increase in N-acetyltransferase activity in intact rats subjected to swimming stress

As discussed in the Introduction, it has been suggested that the enhanced increase in pineal gland N-acetyltransferase activity in chemically sympathectomized rats subjected to immobilization or hypoglycemia stress might be due to supersensitivity

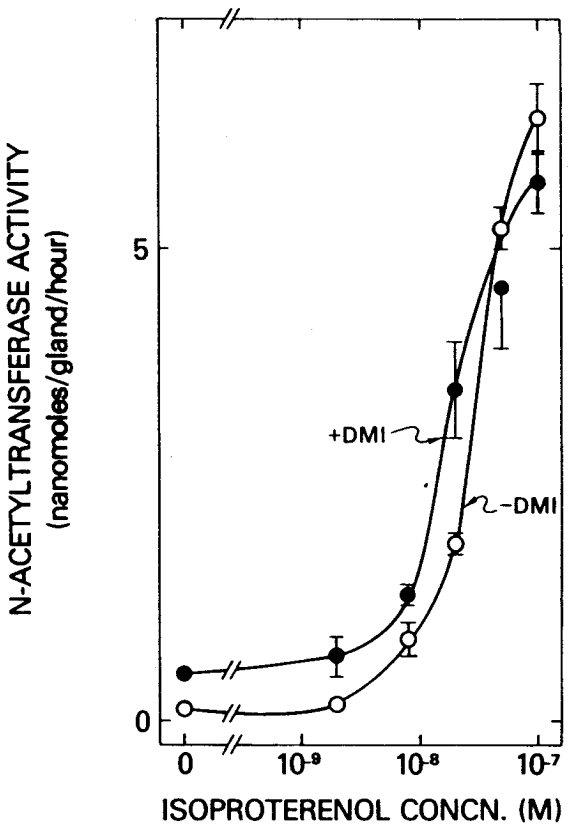

FIG. 8. Effect of DMI on the dose-response curve for isoproterenol stimulation of N-acetyltransferase activity in pineal glands which had been cultured for 5 h following removal from intact rats. Pineal glands taken from intact rats were cultured for 5 h then treated for 2.5 h with isoproterenol (10^{-7}M to 2×10^{-9}M) either alone (○) or in the presence of 10^{-6}M DMI (●). DMI treatment began 5 min before addition of isoproterenol. Each point represents the mean (±SE) of the enzyme activity determined in 4 separate glands.

of the gland to β-adrenergic stimulation (5). One method of producing supersensitivity is prolonged exposure to constant light (16). After 22 days constant light, however, the increase in N-acetyltransferase activity in response to swimming stress was only 2.4-fold greater than the response seen after 8 h of light and was less than one-third the response seen in stressed SCGX rats which had been maintained on a normal lighting schedule (Table 1) (6).

5. *Relative effects of different stressful treatments on N-acetyltransferase activity in intact and SCGX animals*

The effects of several forms of stress were examined (Table 2). Neither cold (4 C), immobilization, nor swimming produced a greater than 5-fold increase in enzyme activity in intact animals. In SCGX animals, in which enzyme activity was elevated about 5-fold in this experiment, each stressful treatment produced a 40- to 50-fold increase in N-acetyltransferase activity relative to unstressed intact animals.

TABLE 1. The effect of constant light on the response of pineal gland N-acetyltransferase activity in rats subjected to swimming stress

Treatment			
Time in constant light	Swimming stress	SCGX	N-Acetyltransferase activity (nmol/gland/h)
8 h	–	–	0.29 ± 0.10
	+	–	1.01 ± 0.22
19 h	+	–	1.07 ± 0.19
11 days	+	–	1.43 ± 0.19
22 days	–	–	0.14 ± 0.08
	+	–	2.41 ± 0.57†
8 h*	–	+	0.44 ± 0.06
	+	+	7.78 ± 1.02‡

Rats were caged in groups of 6 and maintained in constant light for the time indicated prior to a 2.5 h swimming stress. SCGX rats were maintained on a normal lighting schedule (*L*:D *14*:10).

* Data taken from Table 1, Ref. 6.

† Significantly greater than response to swimming stress in intact animals after 8 h light, $P < 0.05$.

‡ Significantly greater than all other groups, $P < 0.01$.

TABLE 2. Relative effects of different stressful treatments in normal and SCGX animals

Treatment		N-acetyltransferase activity (nmol/gland/h)
Stress	SCGX	
—	–	.17 ± 0.021
—	+	.73 ± 0.190*
Cold (4C)	–	.57 ± .295
Cold (4C)	+	8.6 ± .77*
Immobilization	–	.35 ± .106
Immobilization	+	6.5 ± 1.65*
Swimming	–	0.78 ± .193*
Swimming	+	7.2 ± 0.47*

Each stressful treatment was initiated at 1200 h and was maintained for 2.5 h. Immobilization was performed as previously described (5). Animals were housed in individual cages during cold stress. Data are presented as the mean ± SE of the enzyme activity determined in 6 separate glands.

* Significantly greater than unstressed, intact animals, $P < .01$.

6. *Effect of DMI on the increase in N-acetyltransferase activity in intact rats subjected to cold stress*

We determined whether the DMI effect might be seen in association with a form of stress other than swimming (Table 3). Animals treated with DMI (10 mg/kg) and exposed to 4 C for 2.5 h had enzyme values 10-fold greater than animals treated with DMI alone and about 50-fold greater than cold-stressed animals not treated with DMI.

Discussion

The general area of interest in which this study falls is that of the physiological regulation of indole amine metabolism in the pineal gland. Large daily changes in the production of N-acetylserotonin and melatonin and in the concentration of serotonin are regulated by large, up to 100-fold, changes in the activity of N-acetyltransferase (1,2). The activity of this enzyme is regulated by environmental lighting working in conjuction with a precise endogenous clock mechanism of which a major component appears to reside in the hypothalamus, specifically in the suprachiasmatic nucleus (18). Neural connections from this area carry stimulatory signals to sympathetic

TABLE 3. The effect of DMI on cold-stressed animals

Treatment		
Cold stress	DMI (10 mg/kg)	N-acetyltransferase activity (nmol/gland/h)
−	−	0.13 ± 0.031
−	+	1.1 ± 0.420*
+	−	0.18 ± 0.010
+	+	10.9 ± 0.23†

For details see the legend of Table 2.
* Significantly greater than animals not treated with DMI, $P < .01$.
† Significantly greater than all other groups, $P < .01$.

nerve endings in the pineal gland. These stimulatory signals, which are generated only during the dark phase of a light:dark cycle and can be blocked by environmental light at night, cause the release of norepinephrine from the pineal nerve terminals. This results in an increase in N-acetyltransferase activity (1,2).

The large magnitude of changes in pineal N-acetyltransferase, coupled with the extremely rapid nature of these changes, led us to propose that this enzyme is the key to the ability of the pineal gland to provide accurate and reliable measures of the duration of the dark period in the form of melatonin (1). In addition, the inability of an exposure during the day to cause an increase in enzyme activity enhances the reliability of this mechanism. Thus, a report of Lynch *et al.* (5) describing 100-fold changes in N-acetyltransferase activity caused by stress was of some interest to us. If stress could cause changes in N-acetyltransferase activity similar to those which occur at night, one might predict that under natural circumstances the large changes in pineal indole metabolism which we propose occur only at night would occur any time an animal is stressed. This certainly would reduce the reliability of melatonin production by the pineal gland as a measure of the dark period.

The findings of Lynch *et al.* stimulated us to initiate investigations into the effects of stress on pineal N-acetyltransferase. Several important discrepancies between our results and those of Lynch became apparent; these are discussed below in the first portion of our discussion. In the second portion we discuss our data as it bears on the theory that the adrenergic system can play a passive homeostatic role during stress and protect the pineal gland against a major stimulation by circulating catecholamines.

Discrepancies regarding the effects of stress on pineal N-acetyltransferase activity in intact animals

Two major discrepancies between our studies and those of Lynch *et al.* (5) are apparent. The first has to do with the absolute values of N-acetyltransferase activity. We report control rates (0.1 to 0.2 nmol/gland/h) which are 10- to 500-fold higher than those reported by Lynch *et al.* (0.1 to 2.7 pmol/gland/15 min). In addition, the activity we report in stressed denervated animals of 10–20 nmol/gland/h is about 10-fold higher than that reported by Lynch *et al.* (approx. 300 pmol/gland/15 min). This discrepancy in absolute values is due in part to the higher concentration of substrates used in our assay. The concentrations of tryptamine and acetyl-CoA we use are 10 mM and 0.5 mM, respectively; those of Lynch *et al.* are 2.5 mM and 0.028 mM, respectively. Both laboratories use modifications of the assay of Deguchi and Axelrod (13). The concentrations we use were chosen because they are about 4 to 5 times greater than the apparent K_m values for tryptamine and acetyl-CoA as determined in preliminary experiments using homogenates of pineal glands obtained from adrenergically stimulated rats (Parfitt, A. G., and D. C. Klein, unpublished results). Furthermore, we sonically disrupt pineal glands in the assay mixture in polyethylene assay tubes whereas Lynch *et al.* (5) homogenized in buffer in ground-glass tubes. We have found that even transient contact with a ground-glass surface in the absence of 0.5 mM acetyl CoA causes a marked reduction of N-acetyltransferase activity (Parfitt, A. G. and D. C. Klein, unpublished observation). Finally,

we have also found that N-acetyltransferase is highly unstable in the absence of acetyl CoA or related compounds; the concentration of acetyl CoA that provides 50% protection of N-acetyltransferase activity in glands homogenized in glass is approximately 0.3 mM (19).

The second discrepancy has to do with the relative effects of stress in the intact animal. Using several forms of stress, including cold exposure, immobilization, and swimming, we have not been able to detect a consistent increase in pineal N-acetyltransferase activity in intact animals of more than 10 times (Table 2) control values. This is equivalent to a change of no more than 1 nmol/gland/h. In contrast, using immobilization stress, Lynch *et al.* report an increase over control values of 117-fold (5), equivalent to about 0.14 nmol/gland/h. The discrepancy in the relative increase in enzyme activity may be a reflection of a different pretreatment. Lynch *et al.* used animals which had been starved for 15 h and kept in constant light for 48 h prior to stress. We chose to study a less sensitized animal; our animals were fed *ad libitum* and were housed under diurnal lighting conditions.

Another possible explanation for the discrepancy between the various stress: control ratios in intact animals reported here and that reported by Lynch *et al.* relates to the fact that in the latter case the ratio is based on a very low control value (0.1 pmol/gland/15 min). Using data provided (5) we calculate that only 6 cpm must be measured to obtain this value. If the high background (200–300 cpm) inherent in the original procedure (13) prevails in the modification used by Lynch *et al.* (5), low values of enzyme activity might be unreliable. This problem of high background was overcome in our laboratory by substituting a neutral buffer for the alkaline buffer in the original (13) product-extraction procedure and using chloroform for the organic extraction; a background of 30–40 cpm results. Support for the suggestion that the assay used by Lynch *et al.* might be unreliable for low values of N-acetyltransfefase is obtained from the report by Lynch *et al.* which presents three experiments in which control values varied by a factor of 27-fold (5).

Alternatively, the 117-fold increase in N-acetyltransferase activity might be an accurate reflection of changes which we cannot detect in our assay because of inherent insensitivity or higher substrate concentrations. High substrate concentrations might mask a hypothetical stress-induced shift in the K_m of the enzyme for either substrate. A shift in the K_m for acetyl CoA, for example, from 30 μM to 3 μM might occur. If this were the case, the assay used by Lynch *et al.* could reflect such a change because the concentration of acetyl CoA is 28 μM in their assay. Our assay would not detect such a hypothetical change because the enzyme would be completely saturated in both cases. The meaningful direct examination of this explanation is, however, presently impracticable because the relatively pure preparation of the enzyme needed for the experiments has not been obtained.

The role of nerve endings in protecting against stress-induced changes in N-acetyltransferase activity

In spite of the discrepancies discussed above, our studies and the studies by Lynch *et al.* clearly indicate that denervation, accomplished either through chemical or surgical procedures (5,6), results in about a 10-fold increase in the N-acetyltransferase response to stress. In our case this results in stress:control values of 40–80; in the case of Lynch *et al.*, it results in stress:control values of 300–1000. This increased response is clearly due in part to supersensitivity to adrenergic agonists (10).

In addition, we had suspected that the increased response in denervated glands may also, in part, have been due to the loss of $uptake_1$ associated with denervation. $Uptake_1$ may have been important in preventing an acute increase in N-acetyltransferase activity in response to a stress-induced in-

crease in circulating catecholamines. If this were so, inhibition of this process should result in an increase in the N-acetyltransferase response to stress. Previous studies using phentolamine, which in addition to being an α-adrenergic blocking agent, is a weak inhibitor of uptake$_1$ (20), suggested to us that this was the case (6). We found that the administration of phentolamine to stressed animals resulted in an increase in pineal N-acetyltransferase activity. In the present experiments more potent and specific inhibitors of uptake$_1$, DMI and IMIP, were used. Stressed animals which were treated with either of these drugs exhibited a larger N-acetyltransferase response. These observations are consistent with the hypothesis that uptake$_1$ plays a protective role in the pineal gland by sequestering catecholamines and maintaining low concentrations in the pineal extracellular space. Direct measurement of this parameter, impossible with present techniques, would be of great value.

Nonpharmacological evidence is also available which supports our hypothesis regarding the relative importance of uptake$_1$ and supersensitivity. Pineal glands can be made supersensitive by methods which do not remove nerve endings (21,22). These methods include decentralization of the superior cervical ganglia or prolonged exposure to light. In neither case did stress produce as large an increase in N-acetyltransferase activity as that seen in denervated pineal glands (6; Table 1; Figs. 4 and 5).

Although it appears likely that the effects of DMI and IMIP are mediated primarily through their well characterized effect as inhibitors of uptake$_1$, other possibilities should be examined. DMI might act only by causing a large increase in the levels of circulating catecholamines. However, we found that DMI failed to potentiate the stress-induced increase in N-acetyltransferase activity in SCGX animals (Fig. 5). In addition, IMIP did not stimulate N-acetyltransferase in unstressed intact rats and produced only a minor increase in SCGX animals, relative to the maximum response. A large response might have occurred if there were both a large increase in circulating catecholamines and if uptake$_1$ in the pineal gland was ineffective. We also found in organ culture that in the presence of a fixed concentration of EPI, DMI and a clear sensitizing effect (Fig. 6) similar to that seen *in vivo* (Fig. 4). Thus, although an increase in circulating catecholamines in response to DMI treatment might occur, our evidence does not support the proposal that DMI is acting to increase the pineal N-acetyltransferase response primarily by increasing circulating levels of catecholamine.

DMI, which is known to lower serotonin levels in pineal nerves (23,24), could also have acted *via* a serotonin mechanism to increase the sensitivity of the adrenergic receptor. This does not, however, appear to be the case. DMI, which altered the *in vitro* response to EPI treatment (Fig. 6a), had no effect on the *in vitro* response to isoproterenol (Fig. 8). Presumably, isoproterenol and EPI act through the same adrenergic mechanism in the pinealocyte to increase N-acetyltransferase activity. EPI, not isoproterenol, however, is taken up by nerve endings (17). This experiment would indicate that DMI does not alter the adrenergic sensitivity of pinealocytes to adrenergic stimulation through a mechanism involving compounds contained in nerve endings, including serotonin.

Another explanation for the effect of DMI is that it acts *via* other transmitters in the pineal gland. No available evidence, however, supports this. We have recently found that gamma-aminobutyric acid, which is found in the pineal gland, does not alter the dose response relationship for norepinephrine and N-acetyltransferase activity in either denervated pineal glands or glands with nerve endings (Mata, M., B. Schrier, D. C. Klein and C. Y. Chiou, unpublished results). Furthermore, there is no evidence that acetylcholine is either found or can be made in the pineal gland (25); this precludes the possibility that DMI is acting *via* a cholinergic mechanism.

We believe that the best explanation of

our observations is that the response of pineal N-acetyltransferase activity to stress is regulated by both post-synaptic sensitivity and pre-synaptic uptake of catecholamines. This is consistent with the elegant model of adrenergic sensitivity originally described by Trendelenberg (26).

The importance of the pre-synaptic uptake mechanism in modulating a stress response and protecting against non-transsynaptic adrenergic stimulation appears to have gone unnoticed in the literature. It is known by the classic work of Cannon that the sympathetic nervous system usually stimulates sympathetically innervated tissues during stress. Apparently this effect is relatively minor in the intact pineal gland, which does receive heavy sympathetic innervation. It would appear that in the pineal gland the sympathetic system plays more of a passive homeostatic role during stress. It will be valuable to determine if the pineal gland is unique in this regard or if sympathetic innervation in other tissues plays a similar role during stress. Perhaps the relative importance of this mechanism will be determined in a large part by the relative amount of innervation a tissue receives. Heavily innervated tissues might be afforded the most protection by uptake$_1$ and respond minimally to circulating catecholamines.

As a final note it should be mentioned that uptake$_1$ would also appear to enhance the reliability of the pineal gland as a precise device which, in the form of melatonin production, provides a measure of the duration of the dark period (1,27). Uptake$_1$ would tend to protect against large stress-induced changes in pineal indole metabolism during daylight hours when this parameter is normally low.

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